PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: 1654
JAMES J. BENEDICT ET AL.	Examiner: Jeffrey E. Russel
Serial No.: 09/748,038	Attorney Docket: 2103.013900/RFE (SBI-042-CIP)
Filed: December 22, 2000	
For: METHOD OF PROMOTING NATURAL BYPASS	CUSTOMER NO. 45488

DECLARATION OF RAMA AKELLA, Ph.D.
UNDER 37 C.F.R. 1.131

Rama Akella, Ph.D., states as follows:

1. I am one of the inventors of the above-identified patent application.

2. Exhibit 1 is a copy of a research agreement between Sulzer Innotec, Ltd., a corporate predecessor of the present assignee of this patent application, and Columbia University, in which Dr. Daniel Burkhoff of Columbia was designated as the principal investigator of a research project that began before March 31, 2000. The research agreement was amended prior to March 31, 2000. Exhibit 2 is a copy of a research agreement between Sulzer Biologics, Inc., a corporate predecessor of the present assignee of this patent application, and Columbia University, in which Dr. Daniel Burkhoff of Columbia was designated as the principal investigator of a research project that also began before March 31, 2000. Exhibit 3 is a copy of Roethy et al., J. Pharma. Exp. Thera. 299:494-500 (2001), a paper of which I
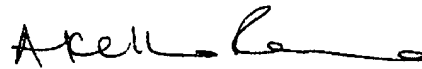
was a coauthor and Dr. Burkhoff was the correspondence addressee. Exhibit 3 reports results from the studies established by the research agreements of Exhibits 1-2.

3. I was an employee of Sulzer Innotec, Ltd., or its corporate successors as of the effective dates of the research agreements presented in Exhibits 1-3. In my employment, I communicated with Dr. Burkhoff on an ongoing basis about the projects covered by the research agreements.

4. Turning to Exhibit 1, its original purpose is given at Attachment IV, Protocol, paragraph 2, Purpose, which states: "The purpose of this study is to determine whether intramyocardial injections of BP can induce angiogenesis, increase blood flow and improve cardiac function in ischemic myocardium." The study design involved the induction of angiogenesis in canine myocardium (Study Design). Experimental details are given in Attachment IV, Protocol.

5. The successful induction of angiogenesis according to the protocol is indicated by Exhibit 1, Research Agreement Amendment, p. 1, paragraph 4.A: "The purpose of these changes is to provide proof of safety for myocardial angiogenesis with ... Bone Protein... in vivo."

6. The success of the protocol of Exhibit 1 is also indicated by Exhibit 2, Protocol, paragraph 2, Purpose, which states: "The purpose of this study is to answer several basic questions related to previous canine studies with the Bone Protein (BP). One, determine the optimal Povidone concentration and molecular weight for delivery of BP via intramyocardial injections in order to induce angiogenesis in the myocardium." The study design involved the induction of angiogenesis in canine myocardium (Study Design and Experimental Procedure).

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7. Exhibit 3 reports results from the pilot studies and efficacy studies of the induction of angiogenesis in canine myocardium established by the research agreements of Exhibits 1-2. Exhibit 3, Figures 2E-H shows relatively large and apparently mature vessels arose within six weeks of injection with GF_m (a.k.a. BP, Exhibit 1, Protocol, paragraph IV). Figures 4-5 show angiograms also indicating large vessel growth in experimental animals but not in control animals.

8. As stated in the Purposes and Study Designs of Exhibits 1-2 and demonstrated by the results given in Exhibit 3, the other inventors and I conceived of, tested, and showed success for a method of promoting natural bypass in a mammal (in this example, dog) comprising administering to the mammal a mixture of proteins derived from ground bone (in this example, BP) prior to March 31, 2000.

9. Our tests were performed in the United States of America.

10. I declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: June 13, 2005 _____

Rama Akella, Ph.D.

Serial No. 09/748,038
Declaration of Rama Akella

RESEARCH AGREEMENT AMENDMENT

1. The Research Agreement, █████████████, (Attachment I) entered into between Sulzer Innotec Ltd. ("Sulzer Innotec") and the Trustees of Columbia University ("Columbia University"), as amended by the letter amendments dated █████████ (Attachment II) and █████████ (Attachment III), is hereby further amended in order to: (1) assign the Research Agreement, as amended, from Sulzer Innotec Ltd. to Sulzer Biologics Inc., (2) modify the preclinical study protocol to conform with suggestions from the U.S. Food and Drug Administration; and (3) provide an agreed upon amended study cost.

2. The Research Agreement, as amended, is hereby assigned by Sulzer Innotec Ltd. to Sulzer Biologics Inc. and Sulzer Biologics agrees to be bound by the terms thereof.

3. ██ The initial study cost of USD █████████ was paid in full by Sulzer Innotec Ltd., and by Sulzer Innotec's affiliate, Sulzer Carbomedics Inc., 1300 East Anderson Lane, Austin, Texas 78752 ("Sulzer Carbomedics").

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4. Changes to the protocol (the complete modified study protocol is included as Attachment IV):

   ### A. Purpose

   The purpose of these changes is to provide proof of safety for myocardial angiogenesis with ProVasc™ (Bone Protein) in vivo. ███

   ### B. Study Groups

   Dosage has been changed to equal human and 10 times human doses in order to evaluate safety. Twenty-one (21) animals. Total animals in the study is increased by one to allow for three groups of n=7 as opposed to two groups of n=10 as originally stated.

   1.) Control, povidone   n = 7
   2.) 3 mg ProVasc™ (Bone Protein)   n = 7
   3.) 30 mg ProVasc™ (Bone Protein)   n = 7

C. Safety Evaluation

1. Blood and Urine Tests

A standard array of tests will be performed on blood and urine obtained prior to ProVasc™ administration and again 3 and 6 weeks later. The list of tests is summarized in Appendix 1. ███████████████████████████████████████

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████████████████████████████████████

2. Histology

Standard pathologic and histologic techniques will be used to obtain representative tissue samples from a variety of organs to provide safety information concerning the effects of ProVasc™. Examinations will be performed by observers blinded to treatment group. Findings will be recorded on a standardized reporting form. All tissue samples will be fixed in 10% neutral buffered formalin over night and routinely dehydrated and embedded in paraffin. Serial sections, 4-5 μm in thickness will be cut and stained with Masson's trichrome procedure to evaluate the general morphology.

i. Heart: Three samples from each heart will be examined (with the remainder of the heart submitted for microsphere analysis). Trichrome stained sections will be examined in order to easily identify fibrotic regions which are typically observed at sites of growth factor injections. The dimensions of these regions will be documented. However, the clinical significance of these regions will not be obtained from this histologic characteristics; rather the significance (or lack there of) will be obtained through the echocardiographic studies described above.

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ii. Other organs: **Lungs, liver, both kidneys and brain** will be examined grossly after sectioning in ~1cm slices. If no lesions are identified, random samples will be selected for histologic examination. In addition, samples of small intestine and spleen will be obtained and examined histologically.
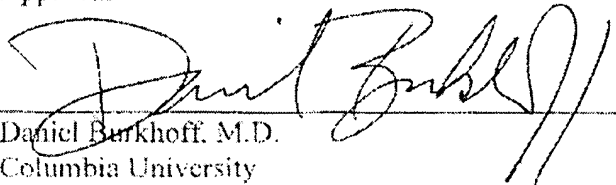
3. Impact

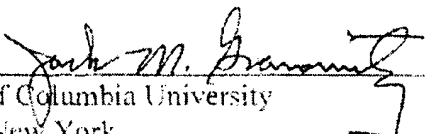The surgical procedures will remain the same.

4. Validity

Except as specifically stated herein, all other terms and conditions of the signed Research Agreement, as previously amended, remain unchanged.
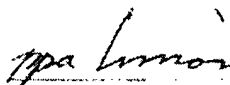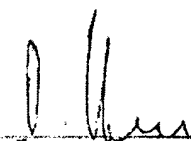
Approval

Daniel Burkhoff, M.D.
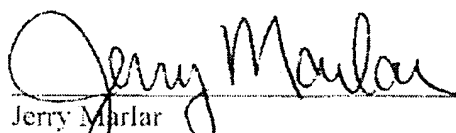Columbia University
Principal Investigator

_____
Date

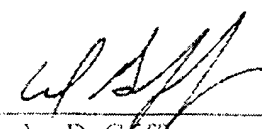The Trustees of Columbia University
In the City of New York

_____
Date

Albert Simon
Head of Infonet, Sulzer Innotec ~~Ltd~~ *Vice-President*
Sulzer Markets and Technology Ltd.

Dr. O. Jten

_____
Date

Jerry Marlar
President, Sulzer Biologics Inc.

_____
Date

Charles D. Griffin
Vice President/General Manager, Sulzer Carbomedics Inc.

_____
Date

BUDGET TO RESEARCH AGREEMENT AMENDMENT

APPENDIX I

List of Blood Tests to be Performed Prior to, 3 Weeks and 6 Weeks After Exposure to ProVasc™ or Placebo

Hematologic Tests:
    activated partial thromoplastin time
    blood cell morphology
    hematocrit
    hemoglobin
    mean corpuscular volume
    platelet count
    prothrombin time
    red blood cell count
    reticulocyte count
    white blood cell count (total, absolute and percent differential)

Blood Biochemistry:
    alanine aminotransferase
    albumin
    alkaline phosphatase
    aspartate aminotransferase
    blood urea nitrogen
    calcium
    cholesterol
    chloride
    creatinine
    globuline (calculated)
    glocose
    phosphorous
    potassium
    sodium
    total bilirubin
    total protein
    triglycerides

Urinalysis:
    appearance
    bilirubin
    blood
    glucose
    ketones
    microscopy of cetnrifuged sediment
    nitrites
    pH
    protein
    specific gravity
    urobilinogen

Research Agreement

RESEARCH AGREEMENT

This Agreement is entered into between Sulzer Innotec Ltd. ("Sulzer Innotec"), Postfach, CH-8401 Winterthur, Switzerland and The Trustees of Columbia University in the City of New York on behalf of Columbia Innovation Enterprise, ("Columbia or University")/630 West 168th Street, PH-15 1540 Center, New York, NY 10032.

1. Statement of Work. The University agrees to use its reasonable efforts to perform the research project (the "Project") described in the Protocol attached to and made part of this Agreement (the "Protocol"). The Project will be performed at the University. The University will provide personnel, facilities and resources as required to accomplish the analysis outlined in the Protocol. In the event of a conflict between the terms of this agreement and the Protocol, this agreement shall govern. Notwithstanding any term of this Agreement or the attached protocol, University does not promise, warrant, guarantee or agree that it will achieve any particular study result, or that the Project will result in any approvals from the U.S. Food and Drug Administration.

2. Principal Investigator. The study will be supervised by Dr. Daniel Burkhoff (PI) who hereby accepts responsibility for the conduct of the Project and adherence to this Agreement and to the Protocol. Dr. Burkhoff shall perform the services under this Agreement. If, for any reason, Dr. Burkhoff is unable to continue to perform the services under this Agreement and a successor acceptable to both the University and Sulzer Innotec is not available, this Agreement may be terminated by either party forthwith upon written notice.

3. Period of Performance. The period of performance will begin on ██████████ and will continue until the Project outlined by the Protocol is completed or otherwise terminated as outlined by Paragraph 5 of this Agreement.

4. Payment. ████████████████████████████████████

███

5. Termination. ██████████████████████████████████

6. Confidentiality.

7. Reports.

8. Publication and Copyrights.

9. Use of Names.

10. Invention. As long as Sulzer Innotec is not in default under any provision of this Agreement, University agrees that any and all inventions (which terminology includes, without limitation, discoveries, designs, methods, processes, products, conceptions, innovations, improvements, enhancements, and the like) relating to the bovine-derived combination of growth factors collectively known as bone protein (BP; described in US Patents 5,290,763 and 5,371,191) whether patentable or not, made, developed, perfected, devised, conceived or first reduced to practice by University, either alone or with others, during the term of this Agreement and in the course of the University's work for Sulzer Innotec will be the sole and exclusive property of Sulzer Innotec. University will hold each and every such invention in a fiduciary capacity for Sulzer Innotec's benefit and promptly disclose to Sulzer Innotec in writing complete information thereon. University agrees to assist Sulzer Innotec in any and all matters pertaining to obtaining patents on such inventions and to execute documents including but not limited to assignments to Sulzer Innotec which Sulzer Innotec deems necessary for that purpose, when and as requested

by Sulzer Innotec. If any such activities are performed after the termination of this Agreement, Sulzer Innotec will pay University at a mutually agreed rate for reasonable time spent in the performance thereof and will reimburse University for reasonable and necessary out-of-pocket expenses actually incurred for or in connection with such activities. Any other discoveries or inventions developed or conceived solely by the University will be the sole property of the University.

11. Independent Contractor.
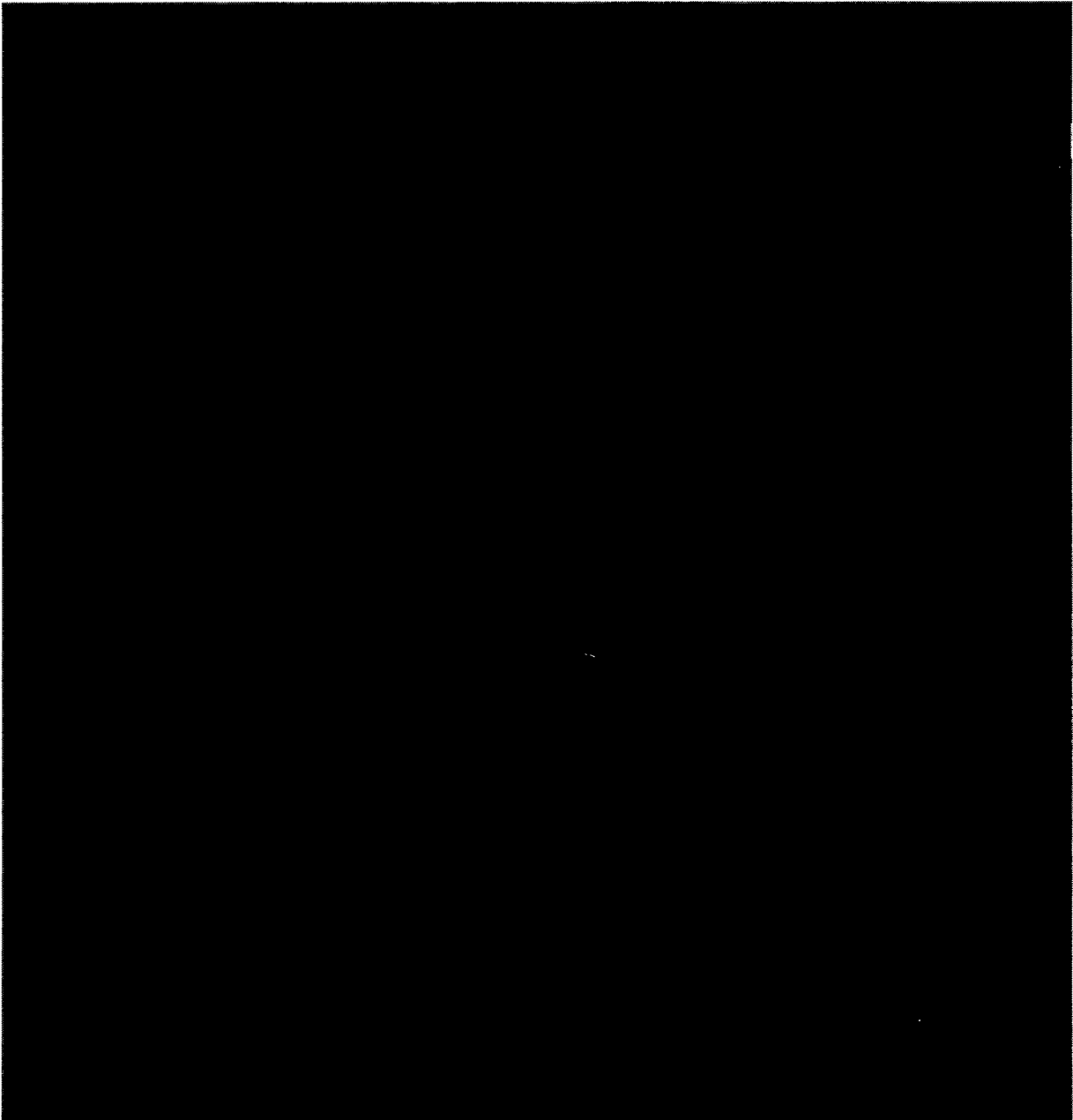
12. Governing Laws.

13. Modifications.

14. Notices.

SULZER*INNOTEC*

15. Indemnity and Insurance.

Executed as of the date first above written.

SULZER INNOTEC, LTD

By: _____

Printed Name: _____

Title: _____

Winterthur ▮▮▮▮▮▮

Agreed:

PRINCIPAL INVESTIGATOR

Daniel Burkhoff, M.D., Ph.D.

The Trustees of Columbia University
in the City of New York

By: _____

Printed Name: _____
Jack M. Granowitz

Title: _____
Executive Director
Columbia Innovation Enterprise

SULZER *INNOTEC*

PROJECT BUDGET
(In U.S. Dollars)

SULZERMEDICA

Sulzer Carbomedics Inc.

1300 East Anderson Lane
Austin, Texas 78752-1793

Phone 512 435-3200
Fax 512 435-3050
WATS 800 845-1678 US and Canada

Executive Director
Columbia Innovation Enterprise
Columbia University
500 West 120th Street, mc2206
Engineering Terrace, Suite 363
New York, NY 10027
USA

Subject: Agreement between Columbia University and Sulzer Innotec Ltd., Ref. No.
▮▮▮▮▮▮▮▮▮▮

Ladies and Gentlemen

We hereby declare that Sulzer Carbomedics Inc., of 1300 East Anderson Lane, Austin, Texas 78752, a
company of Sulzer Medica, is affiliated with Sulzer Innotec Ltd.

Yours faithfully,

Sulzer Carbomedics Inc.

Charles D. Griffin
Vice President/General Manager

Copy to: Sulzer Innotec Ltd.

ATTACHMENT II

SULZER*INNOTEC*

Sulzer Innotec Ltd
P.O. Box
CH-8401 Winterthur
Switzerland

Phone 052/262 21 21
Telefax 052/262 00 15

Executive Director
Columbia Innovation Enterprise
Columbia University
500 West 120 th Street, mc 2206
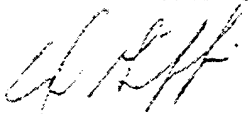Engineering Terrace, Suite 363
New York, NY 10027

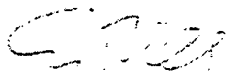**Subject: Project Agreement,** ████

Ladies and Gentlemen

We hereby declare that Sulzer Carbomedics Inc., of 1300 East Anderson Lane, Austin, Texas 78752, a company of Sulzer Medica, is an affiliated company with Sulzer Innotec Ltd.
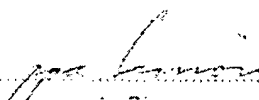
████████████████████████████

Please consider this letter as a legal addendum to the Project Agreement and confirm it's receipt by return mail.
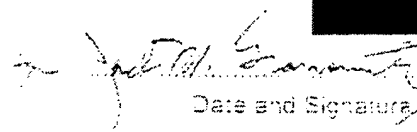
Yours faithfully

Sulzer Innotec Ltd.

Dr. E. Rikli
President a.i.

A. Simon
Head of Infonet

Date:

Receipt acknowledged
Columbia University

████

Date and Signature

Copies to Sulzer Carbomedics Inc.

ATTACHMENT III

PROTOCOL

1. TITLE

In vivo evaluation of Bone Protein (BP: Sulzer Orthopedics Biologics Inc.) in a canine myocardial ischemia model.

2. PURPOSE

The purpose of this study is to determine whether intramyocardial injections of BP can induce angiogenesis, increase blood flow and improve cardiac function in ischemic myocardium.

3. LABORATORY PRACTICE

This study will be conducted in accordance with the Columbia University's Standard Operating Procedure.

4. TEST MATERIAL

Bone Protein (BP) is a proprietary mixture of growth factors derived from bovine bone and manufactured by Sulzer Orthopedics Biologics Inc. (Denver, CO).

5. STUDY DESIGN

Twenty (20) adult mongrel dogs of either sex, weighing 21-26 kg, will be anesthetized with intravenous injection of thiopental sodium (15mg/kg) and maintained with 0.5-2.0% inhaled isoflurane. A left thoracotomy is performed through the fifth intercostal space using sterile technique. All visible epicardial collaterals connecting LAD diagonals to circumflex or right coronary arteries will be ligated with a 4-0 stitch to minimize collateral flow to the LAD territory and an ameroid constrictor will be placed on the LAD proximal to the first diagonal branch. A left atrial cannula will be placed and tunneled subcutaneously; this will be used for subsequent microsphere injections. After completing the procedure, the chest will be closed in layers and the animal will be allowed to recover.

After six weeks, with the animal in a conscious sedated state, regional function and coronary blood flow will be assessed at baseline and during adenosine stress by

echocardiography and the microsphere method, respectively. In order to assess the blood flow capacity of the remaining collateral circulation, 1 ml of mixed colored microsphere solution (15 μm diameter, in saline suspension) are injected into the left atrium through the previously placed catheter. To assess collateral flow during stress, microspheres of a different color are injected following intravenous adenosine infusion. The rate of infusion is titrated as in previous studies to create a 20% mean fall in arterial pressure. Blood samples are obtained during microsphere injections by a constant rate withdrawal (7 ml/min) from the femoral artery using a syringe pump. Concomitant with the microsphere injections, echocardiography will be performed as a means of assessing cardiac function under baseline and stress conditions. Following completion of these diagnostic procedures, the animals will be subjected to a second survival surgery. Animals will be divided into two groups. The first will receive intramyocardial injections of growth factor in the ischemic region, and the second will receive control, carrier only injections. After completing the procedure, the chest will be closed in layers and the animal will be allowed to recover.

In order to provide an index of cellular proliferation at multiple time points following growth factor injection, bromodeoxyuridine (BrdU, 25 mg/kg, Sigma, St. Louis, MO) will be administered subcutaneously on days 2, 4, 6, 8, 10, 14 and 21 after the second surgery.

Six weeks following myocardial injection with the animals in a sedated state, the same protocol of echocardiography and microsphere infusions under resting and adenosine stress conditions described above will be performed using microspheres of two additional colors. Euthanasia (pentobarbital, 100 mg/kg) will then be performed and the heart will be explanted. The coronary arteries will be injected with radiocontrast agent and an x-ray will be taken at two orthogonal views. The heart will then be cut into small (approximately 1g) transmural samples and submitted for microsphere and immunohistochemical analysis.

Microspheres Analysis. Retrieval and quantitative analysis of the microspheres are performed exactly as described previously. In brief, tissue samples are digested and the microspheres are retrieved by filtration of the digestate. The dye on the microspheres is then itself digested into solution using dimethylformamide and the photometric absorption of the resulting sample is measured by a diode array spectrophotometer (model 8452A, Hewlett-Packard Co., Palo Alto, CA). The composite spectrum is then resolved at the peak frequencies into the contributions from the individual colored microspheres using a matrix inversion technique. The number of microspheres in each sample is calculated according to the optical density at the wave length corresponding to each dye color using standardization curves generated from known quantities of microspheres from the same batch of spheres. Regional myocardial blood flow (RMBF, ml/min/g) is calculated using the following equation: $RMBF = (Fref/Msample) \times$

(ODsample/ODref), where Fref is the rate at which arterial blood is withdrawn from the femoral artery (i.e., of the reference sample, which is alway 7 ml/min in our studies). ODref is the optical density of the dye solution obtained from this reference sample, Msample is the mass of the respective myocardial sample, ODsample is the optical density at the corresponding wavelength of the dye solution obtained from the myocardial sample.

Immunohistochemical Analysis. Samples are fixed in 10% neutral buffered formalin over night and routinely dehydrated and embedded in paraffin. Serial sections, 4-5 microns thick, are cut and stained with Masson's trichrome procedure to evaluate the general morphology of the myocardium. Sister sections are stained using standard immunohistochemical techniques with antibodies against bromodeoxyuridine (BrdU), PC10 proliferating cell nuclear antigen (PCNA), Factor VIII, and alpha smooth muscle actin (SMA) using standard techniques.

6.   EXPERIMENTAL PROCEDURE

Twenty (20) animals will be included in the study. Six weeks following induction of ischemia by ameroid constrictor placement, the animals will be divided into two treatment groups.

1) 10 myocardial injections, each 0.1 cc, 0.5 mg/ml BP in povidone carrier (500 µg injected, total)
2) 10 myocardial injections, each 0.1 cc, povidone carrier alone

Following growth factor or control myocardial injections, the animals will be allowed to recover. Final clinical endpoints assessment will be performed six weeks following growth factor administration.

7.   DURATION OF STUDY

Nine (9) months is the estimated duration of the study including all surgical procedures, diagnostic tests and histological evaluation.

8.   PERFORMANCE EVALUATION

Clinical endpoints include microsphere analysis to quantify myocardial blood flow at rest and during stress, echocardiography to evaluate cardiac function, and histological analysis to visualize myocardial blood vessel morphology.

9.   **REPORTING**

Twelve (12) months following the start date. Columbia University will provide Sulzer Innotec a final report including all diagnostic and histological data collected during the study.

10.   **TEST FACILITY**

Columbia University
College of Physicians and Surgeons
630 W. 168th St.
New York, NY 10032
USA

11.   **SPONSOR**

Sulzer Innotec, Ltd.
~~Sulzer Carbomedics Inc.~~
~~1300B East Anderson Lane~~
~~Austin, TX 78752~~
~~USA~~

12.   **ESTIMATED START DATE**

13.   **ESTIMATED COMPLETION DATE**

CONFIDENTIAL

SULZER *INNOTEC*

PROTOCOL APPROVAL

TEST FACILITY

Columbia University

_____
Study Director

[Date — redacted]
Date

SPONSOR

Sulzer Innotec. Ltd.

_____
Program Director

_____
Date

PRE-CLINICAL STUDY PROTOCOL

Title:

In Vivo Evaluation Of Growth Factor Mixture (GF$_m$) Derived From Bovine Bone (Sulzer Orthopedics Biologics Inc.) In A Canine Model Of Myocardial Ischemia

Sponsor:

Sulzer Innotec, Ltd.
Sulzer Carbomedics Inc.
1300B East Anderson Lane
Austin, TX 78752

PROTOCOL

I. TITLE

In vivo evaluation of growth factor mixture (GF_m) derived from Bovine Bone (Sulzer Orthopedics Biologics Inc.) in a canine model of myocardial ischemia

II. PURPOSE

The primary purpose of this study is to determine whether intramyocardial injections of GF_m are safe in a canine model of chronic myocardial ischemia. Safety will be assessed by examining a standard array of blood chemistry and hematologic parameters, resting ventricular function assessed by echocardiography and through histologic examination of the heart and other organs. The secondary purpose of this study is to test for beneficial effects of intramyocardial GF_m injections in chronically ischemic myocardium. Benefits will be assessed by determination effects of GF_m treatment on vascular growth, blood flow, coronary artery anatomy and cardiac function.

III. LABORATORY PRACTICE

The use of animals and the species of animal prescribed for this study are consistent with the conduct of studies of this type. The scientific literature and regulatory requirements from various agencies, including the Food and Drug Administration, support the use of animals in studies such as this. All animals involved in the study will receive humane care in compliance with the "Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society (revised 1980) and the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985). All animals will be housed in an American Veterinary Association-approved facility and all animal protocols are approved by Columbia University's Institute of Animal Care and Use Committee.

IV. TEST MATERIAL

Bone Protein (GF_m) is a proprietary mixture of growth factors derived from bovine bone and manufactured by Sulzer Orthopedics Biologics, Inc. (Denver, CO). GF_m will be dissolved in 1% povidone.

V. IDENTIFICATION OF SPONSOR AND TESTING FACILITY

A. Sponsor

Sulzer Innotec, Ltd.
Sulzer Carbomedics Inc.
C/O Barbara Mathews
1300B East Anderson Lane
Austin, TX 78752

B.   Test Facility
Daniel Burkhoff
Columbia University
630 West 168th Street
New York, NY 10032

VI.   Identification of Test System

A.	Species:	dog
B.	Number:	21
C.	Body Weight Range:	20-30 Kg
D.	Sex:	Approximately evenly divided between male and female
E.	Source of Supply:	Team Associates, Dayville CT.
F.	Age:	2-5 years of age
G.	Procedure for identification:	Each animal will be identified by a uniquely numbered collar tag and cage card.

VII.   STUDY DESIGN

A.   Study Type

This study will be a blinded, prospective study to evaluate the safety and potential beneficial effects of intramyocardial injections GF$_m$ in a canine model of chronic myocardial ischemia.

B.   Description of Surgical Methods and Protocol

An overview of the study is provided in Figure 1. The study will be divided into 6 major stages.

1.   **Initial surgery to create a model of chronic ischemia**
Anesthesia will be induced with medazolam and maintained with inhaled isoflurane (1-2%). The animal will be prepared and draped in the usual manner for a left lateral thoracotomy in the 4th or 5th intercostal space. The pericardium will be excised and the heart exposed. The proximal left anterior descending artery (LAD) will be isolated. In order to induce chronic myocardial ischemia,

an ameroid constrictor will be placed on the LAD. In addition, all visible epicardial vessels (i.e., visible vessels connecting between marginal and diagonal branches and between the posterior descending and distal LAD) supplying collateral flow to the LAD territory will be ligated. A plastic tube will be implanted into the left atrium; this will be used to infuse color microspheres to measure regional myocardial blood flow. In addition, a second tube will be placed in the descending aorta; this will be used to withdraw arterial blood during periods of microsphere infusions to provide a reference sample for allowing absolute quantification of blood flow from the microspheres. The free ends of the left atrial and aortic tubes will be tied, led out of the chest through the 7th intercostal space and will be placed subcutaneously between the shoulder blades. A non-absorbent suture will be tied to each line and this will be exposed through a small incision in the skin. This allows easy access to the tubes from their subcutaneous position during the first experiment in the conscious state (see below). The chest will be closed in layers and the pneumothorax will be reduced. The animal will be allowed to recover.

2. **Physiologic assessment of blood flow and myocardial function in conscious dogs to characterize the ischemic state**

    Starting one week after the initial surgery, each animal will be trained to lie quietly on a laboratory table and to become acclimated to the laboratory environment. Between 20-22 days (~3 weeks) following the initial surgery, an assessment of blood flow and myocardial function will be made with the animal in the conscious state. Experiments will be performed at least 4 hours apart, preferably on separate days. With the animals resting quietly on the laboratory table, the ends of the two subcutaneous tubes will be pulled through the preformed incision in the skin. The descending aortic line will be connected to a transducer and arterial pressure and heart rate will be monitored continuously during the experiments. Blood samples will be obtained and these will be submitted for routine analysis as detailed in Section VII.C.1.b. Resting blood flow will be assessed using rapid infusion of colored microspheres (COLOR 1) through the previously placed left atrial line. Just prior to the infusion, withdrawal of arterial blood from the descending aortic line will be instituted at a rate of 7 ml/min using a constant flow pump (Harvard Apparatus) and this will continue for a total of 2 minutes (14 ml). To induce vasodilatory stress, adenosine will be infused at a dose and rate titrated to cause an approximately 20% decrease in mean arterial pressure. After achieving this blood pressure reduction, blood flow will be assessed through infusion of a second color microsphere (COLOR 2). Within the same 20-22 day window, if possible on a different day, resting and stress echocardiography will be performed. These will be performed using protocols to standardize echocardiographic windows and views. Stress will be induced through the use of intravenous dobutamine infused at a maximum rate of 30 μg/kg/min. Since all investigators are blinded as to treatment group, microsphere and

echocardiographic images are obtained and analyzed in a blinded manner. Also, as will be reviewed below, 5 different colored microspheres will be used in each animal through the course of the study; the sequence with which different colors will be used will be randomized among animals.

3. **Baseline coronary angiography and second surgery for intramyocardial injections of GF_m or placebo.**

The dog will be anesthetized using the same anesthetic protocol as for the first surgery. Urine samples will be obtained using a suprapubic cannula and these will be submitted for routine analysis as detailed in Section VII.C.1.b. The right femoral artery will be isolated surgically and a standard left coronary artery catheter will be introduced under fluoroscopic guidance through the artery into the left main coronary artery. Angiography will be performed using standard views to visualize the LAD and diagonal vessels. These images will be recorded on VHS video tape. After completing the angiography, the catheter will be withdrawn, the femoral artery ligated, and the skin incision closed. The chest will be prepared and draped in the usual sterile fashion and the chest opened in the 4th or 5th intercostal space. At this time the animal will be randomly assigned to one of three experimental groups. Group 1 animals will receive intramyocardial GF_m injections at a GF_m concentration of 1 mg/ml, 0.15 ml/injection, 1 injection/cm^2 to the LAD region. Group 2 animals will receive intramyocardial GF_m injections, at a GF_m concentration of 10 mg/ml, 0.15 ml/injection, 1 injection/cm^2. Group 3 animals will receive intramyocardial injections of vehicle (1% Povidone) without GF_m, 0.15 ml/injection, 1 injection/cm^2. Solutions will be prepared by a single investigator who is independent of the group of investigators performing the surgeries and follow-up tests. It is anticipated that each animal will receive between 15 and 20 intramyocardial injections. A shallow stitch with 4-0 (or thinner) prolene will be placed over each injection site so that each site can be identified when the heart is harvested 6 weeks later. After completing injections, the chest will be closed, the pneumothorax will be reduced and the animal will be allowed to recover. The dogs will receive subcutaneous injections of bromodeoxyuridine (BrdU) starting the day before surgery (25 mg/kg), on the day of surgery (15 mg/kg) and days 1, 3, 5, 7, 9, 13 and 20 after surgery (15 mg/kg) as a means of "marking" dividing cells which can be detected using standard histologic techniques.

4. **Physiologic assessment of blood flow and myocardial function 3 weeks after the second surgery**

Between 20 and 22 days (approximately 3 weeks) after the second surgery, blood flow will be assessed in the conscious state only during adenosine stress using the third colored microsphere (COLOR 3). Resting blood flow will not be measured because there are only 5 different colored microspheres; 2 colors will have been used at baseline and 2 will be required at the final time point (see next section). Within the same 20-22 day time window, the resting

echocardiogram will be repeated. The same protocols for performing microsphere infusions, reference blood sample withdrawals and echocardiogram used during the initial evaluations will be employed. Blood samples will be obtained and these will be submitted for routine analysis as detailed in Section VII.C.1.b.

5. **Physiologic assessment of blood flow and myocardial function 6 weeks after the second surgery**
Between 40 and 44 days (approximately 6 weeks) after the second surgery, blood flow will be assessed at rest and during adenosine stress using the fourth and fifth colored microsphere (COLOR 4 and COLOR 5). The resting and dobutamine stress tests will be performed a minimum of 4 hours apart, preferably on different days within the 40-44 day time window. The same protocols for performing microsphere infusions, reference blood sample withdrawals, echocardiograms and dobutamine infusion used during the initial evaluations will be employed. Blood samples will be obtained to measure a host of chemical and hematologic parameters in order to obtain safety data concerning intramyocardial GF_m.

6. **Coronary angiography followed by sacrifice of the animal and procurement of tissue samples**
After completing blood flow and myocardial functional assessments, animals will be anesthetized as described above, urine samples will be collected in the same manner as during the second surgery and angiography will be repeated using the left femoral artery to introduce the coronary catheter. Images will be recorded on VHS tape to be analyzed later. After completing the angiography, the animal will be sacrificed with an overdose of phenobarbital. The heart will be harvested and sectioned in a consistent manner. Three GF_m injection sites (identified by the previously placed epicardial stitch) will be isolated in individual transmural tissue blocks. These will be cut into three approximately equal thickness sections and placed in formalin for fixation. These sections will be taken from the central region of the ischemic territory. The remainder of the heart will be cut into approximately 1 gram tissue blocks, with a careful map of where individual samples were derived (including epicardial, midwall and endocardial location) and these will be submitted for microsphere analysis. In addition, other organs will be harvested and examined histologically for signs of remote tissue effects of GF_m (see section VII.C.1.c).

C. Data Analysis

1. Safety Analysis
Safety of GF_m injections will be assessed by using echocardiography, standard blood tests and histology.

a. Echocardiography

Echocardiography will be performed under a resting, conscious state 3 weeks after implanting the ameroid constrictor and 3 and 6 weeks after the administration of GF_m or placebo injections. These will be performed by a trained echocardiography technician who is blinded to treatment group. Standard echocardiographic windows will be used to visualize the heart in cross section at the mid-papillary level. The studies will be recorded on VHS tape for off-line analysis by a blinded experienced grader. End-diastolic and end-systolic images will be selected by the grader. The endocardial edge of these images will be traced and commercially available software will be used to determine area ejection fraction, regional wall motion and regional wall thickening at standardized sectors of the heart. GF_m will considered to be safe if these measures of global and regional function are not worse in the treatment groups as compared to the control group. Parameter values from dogs in each group will be pooled, with results expressed as a mean±SD. Values will be compared between groups at each time point using analysis of variance with a post-hoc test (Duncan) to identify differences between specific groups.

b. **Blood and Urine Tests**

A standard array of tests will be performed on blood and urine obtained prior to GF_m administration and again 6 weeks later. The list of tests is summarized in Appendix I. The purpose of these tests is to test whether there are any untoward systemic effects of GF_m in this setting. Data will be examined for any changes from normal values at each time points.

c. **Histology**

Standard pathologic and histologic techniques will be used to obtain representative tissue samples from a variety of organs (listed below) from both treatment groups and the control group. These histopathologic evaluations will be done by Dr. Steven Brunnert a Certified Veterinarian Pathologist, who is independent from our group. A copy of his curriculum vitae is included as Appendix II. This will be done to provide safety information concerning the effects of GF_m. Examinations will be performed by observers blinded to treatment group. Findings will be recorded on a standardized reporting form. All tissue samples will be fixed in 10% neutral buffered formalin over night and routinely dehydrated and embedded in paraffin. Serial sections, 4-5 μm in thickness will be cut and stained with Masson's trichrome procedure to evaluate the general morphology.

   i. Heart: Three samples from each heart will be examined (with the remainder of the heart submitted for microsphere analysis). Trichrome stained sections will be examined in order to easily identify fibrotic regions which are typically observed at sites of growth factor injections. The dimensions of these regions will be

documented. However, the clinical significance of these regions will not be obtained from this histologic characteristics; rather the significance (or lack there of) will be obtained through the echocardiographic studies described above. Specifically, it is anticipated that despite the presence of these regions, the echocardiograms will not show any compromise of ventricular function.

ii. **Other organs: Lungs, liver, spleen, small intestine, both kidneys and brain** will be examined grossly after sectioning in ~1cm slices. If any lesions are identified during gross examination, these regions will be sampled for microscopic examination. If no lesions are identified, random samples will be selected for histologic examination. These evaluations will also be performed by Dr. S. Brunnert.

2. Effectiveness Analysis

a. Blood flow

Retrieval and quantitative analysis of the microspheres will be performed exactly as we have done previously. Any tissue sample which contains grossly evident myocardial infarction will be excluded from analysis. In brief, tissue samples are digested and the microspheres are retrieved by filtration of the digestate. The dye on the microspheres is then itself digested into solution using dimethylformamide and the photometric absorption of the resulting sample is measured by a diode array spectrophotometer. The composite spectrum is then resolved at the peak frequencies into the contributions from the individual colored microspheres using a matrix inversion technique. The number of microspheres in each sample is calculated according to the optical density at the wave length corresponding to each dye color using standardization curves generated from known quantities of microspheres from the same batch of spheres. Regional myocardial blood flow (RMBF, ml/min/g) for each color is calculated using the following equation: $RMBF = (F_{ref}/M_{sample})(OD_{sample}/OD_{ref})$, where F_{ref} is the rate at which arterial blood is withdrawn from the femoral artery (i.e., of the reference sample, which is always 7 ml/min). OD_{ref} is the optical density of the dye solution obtained from this reference sample. M_{sample} is the mass of the respective myocardial sample. OD_{sample} is the optical density at the corresponding wavelength of the dye solution obtained from the myocardial sample.

The entire left ventricle will be subjected to microsphere analysis with the exception of 3 transmural samples submitted for histologic analysis. Myocardial samples will be classified as being from an ischemic territory, from a border zone territory or from a normal territory based upon blood flow during adenosine stress just prior to the initial surgery.

For this determination, blood flows will be compared to those of the remote circumflex (LCx) territory. The following criteria will be used to subdivide the samples:

Ischemic zone: stress blood flow less than 50% of LCx flow
Border zone: stress blood flow 50-85% of LCx flow
Normal zone: stress blood flow >85% of LCx flow

Data from like zones based upon these stress flow measurements prior to the second surgery will be pooled for all blood flow determinations. Data from animal in the same treatment group will be pooled and expressed as means and standard deviations. Blood flow in different treatment groups will be compared using analysis of variance with a post hoc test to test for differences between specific groups.

b.   **Echocardiography**

Regional and global wall motion, assessed as indicated above during rest and stress will be used to look for functional evidence of improved regional myocardial function. Using a sector analysis, it will be determined whether regional wall motion is preserved better during stress in treated as compared to control groups.

c.   **Angiography**

Paired angiograms (baseline and 6 weeks after treatment) recorded on video tape will be reviewed in a blinded manner for evidence of new collateral vessel formation. A semi-quantitative scoring system will be used for this purpose:

-1 - decrease in number of collateral or intramyocardial vessels
0 - no difference
1 - mild increase in number of collateral or intramyocardial of vessels
2 - marked increase in number of collateral or intramyocardial vessels

The following designations will also be used:

"+" to indicate that the distal LAD fills well via collateral flow
"-" to indicate that the distal LAD does not fill well via collateral flow

d.   **Histology**

Samples will be fixed in 10% neutral buffered formalin over night and routinely dehydrated and embedded in paraffin. Serial sections, 4-5 μm in thickness will be cut and stained with Masson's trichrome procedure to evaluate the general morphology of the myocardium. Sister sections will be stained using standard immunohistochemical techniques with antibodies against BrdU, PC10 proliferating cell nuclear antigen (PCNA), Factor VIII and alpha smooth muscle actin (SMA). Slides will be evaluated by a blinded observer for the presence, size and distance from

injection site of new vessels, identified by SMA positivity and positive BrdU staining. Based on preliminary results, it is expected that large, proliferating vessels will be identified only in the two active treatment groups.

APPENDIX I

List of Blood Tests to be Performed Prior to and 3 and 6 Weeks After Exposure to GF_m or Placebo

Hematologic Tests:
    activated partial thromboplastin time
    blood cell morphology
    hematocrit
    hemoglobin
    mean corpuscular volume
    platelet count
    prothrombin time
    red blood cell count
    reticulocyte count
    white blood cell count (total, absolute and percent differential)

Blood Biochemistry:
    alanine aminotransferase
    albumin
    alkaline phosphatase
    aspartate aminotransferase
    blood urea nitrogen
    calcium
    cholesterol
    chloride
    creatinine
    globuline (calculated)
    glucose
    phosphorous
    potassium
    sodium
    total bilirubin
    total protein
    triglycerides

Urinalysis:
    appearance
    bilirubin
    blood
    glucose
    ketones
    microscopy of centrifuged sediment
    nitrites
    pH
    protein
    specific gravity
    urobilinogen

RESEARCH AGREEMENT

This Agreement is entered into between Sulzer Biologics, Inc. ("Sulzer Biologics")/1300 East Anderson Lane, Austin, Texas 78752 and The Trustees of Columbia University in the City of New York, on behalf of Columbia Innovation Enterprise, ("Columbia or University")/630 West 168th Street, PH-1535 East, New York, NY 10032.

1. Statement of Work. The University agrees to use its reasonable efforts to perform the research project (the "Project") described in the Protocol attached to and made part of this Agreement (the "Protocol"). The Project will be performed at the University. The University will provide personnel, facilities and resources as required to accomplish the analysis outlined in the Protocol. In the event of a conflict between the terms of this agreement and the Protocol, this agreement shall govern. Notwithstanding any term of this Agreement or the attached protocol, University does not promise, warrant, guarantee or agree that it will achieve any particular study result, or that the Project will result in any approvals from the U.S. Food and Drug Administration.

2. Principal Investigator. The study will be supervised by Dr. Daniel Burkhoff (PI) who hereby accepts responsibility for the conduct of the Project and adherence to this Agreement and to the Protocol. Dr. Burkhoff shall perform the services under this Agreement. If, for any reason, Dr. Burkhoff is unable to continue to perform the services under this Agreement and a successor acceptable to both the University and Sulzer Biologics is not available, this Agreement may be terminated by either party forthwith upon written notice.

3. Period of Performance. The period of performance will begin on ███████████ and will continue until the Project outlined by the Protocol is completed or otherwise terminated as outlined by Paragraph 5 of this Agreement.

4. Payment. ████████████████████████████████████

5. Termination. ████████████████████████████████████

6. Confidentiality.

7. Reports.

8. Publication and Copyrights.

9. Use of Names.

10. Invention. As long as Sulzer Biologics is not in default under any provision of this Agreement, University agrees that any and all inventions (which terminology includes, without limitation, discoveries, designs, methods, processes, products, conceptions, innovations, improvements, enhancements, and the like) relating to the bovine-derived combination of growth factors collectively known as bone protein (BP; described in US Patents 5,290,763 and 5,371,191) whether patentable or not, made, developed, perfected, devised, conceived or first reduced to practice by University, either alone or with others, during the term of this Agreement and in the course of the University's work for Sulzer Biologics will be the sole and exclusive property of Sulzer Biologics. University will hold each and every such invention in a fiduciary capacity for Sulzer Biologics's benefit and promptly disclose to Sulzer Biologics in writing complete information thereon. University agrees to assist Sulzer Biologics in any and all matters pertaining to obtaining patents on such inventions and to execute documents including but not limited to assignments to Sulzer Biologics which Sulzer Biologics deems necessary for that purpose, when and as requested by Sulzer Biologics. If any such activities are performed after

the termination of this Agreement, Sulzer Biologics will pay University at a mutually agreed rate for reasonable time spent in the performance thereof and will reimburse University for reasonable and necessary out-of-pocket expenses actually incurred for or in connection with such activities. Any other discoveries or inventions developed or conceived solely by the University will be the sole property of the University.

11. Independent Contractor. ███████████████████

███████████████████████████████████

12. Governing Laws. █████████████████████

██████

13. Modifications. ██████████████████████

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14. Notices. ████████████████████████

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15. Indemnity and Insurance. ████████████████████████

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Executed as of the date first above written.

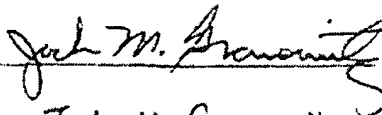SULZER BIOLOGICS, Inc.

By: _____

Printed Name: ___Jerry Marlar___

Title ___President. Sulzer Biologics___

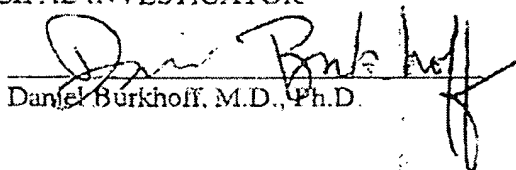The Trustees of Columbia University
in the City of New York

By: _____

Printed Name: ___Jack M. Granewitz___

Title: ___Exe Director/Columbia Innovate Enterprise___

Agreed:

PRINCIPAL INVESTIGATOR

_____
Daniel Burkhoff, M.D., Ph.D.

SULZER *Medica*
Sulzer Biologics

PROJECT BUDGET
(In U.S. Dollars)

PROTOCOL

l. **TITLE**

In vivo evaluation of growth factors and delivery vehicles in a canine myocardial model.

2. **PURPOSE**

The purpose of this study is to answer several basic research questions related to previous canine studies with the Bone Protein (BP). One, determine the optimal Povidone concentration and molecular weight for delivery of BP via intramyocardial injections in order to induce angiogenesis in the myocardium. Two, correlate the angiogenic activity of BP observed in the quail CAM assay with angiogenic activity in the canine myocardium. Three, generate comparative information for the activity of known angiogenic growth factors bFGF and VEGF in this model.

3. **LABORATORY PRACTICE**

This study will be conducted in accordance with the Columbia University's Standard Operating Procedure.

4. **TEST MATERIAL**

Bone Protein (BP) is a proprietary mixture of growth factors derived from bovine bone and manufactured by Sulzer Biologics Inc. (Denver, CO).

5. **STUDY DESIGN**

Ten (10) adult mongrel dogs of either sex, weighing 21-26 kg. will be anesthetized with intravenous injection of thiopental sodium (15mg/kg) and maintained with 0.5- 2.0% inhaled isoflurane. A left thoracotomy is performed through the fifth intercostal space using sterile technique. Animals will receive intramyocardial injections as outlined in the section following. Each injection will be marked with a non-resorbable suture stitch. After completing the procedure, the chest will be closed in layers and the animal will be allowed to recover.

In order to provide an index of cellular proliferation at multiple time points following growth factor injection, bromodeoxyuridine (BrdU, 25 mg/kg, Sigma, St. Louis, MO)

will be administered subcutaneously on days 2, 4, 6, 8, 10, 14 and 21 after the second surgery.

Six weeks following myocardial injection, euthanasia (pentobarbital, 100 mg/kg) will be performed and the heart will be explanted. The coronary arteries will be injected with radiocontrast agent and an x-ray will be taken at two orthogonal views. The heart will then be cut into small (approximately 1g) transmural samples and submitted for immunohistochemical analysis.

Immunohistochemical Analysis. Samples are fixed in 10% neutral buffered formalin over night and routinely dehydrated and embedded in paraffin. Serial sections, 4-5 microns thick, are cut and stained with Masson's trichrome procedure to evaluate the general morphology of the myocardium. Sister sections are stained using standard immunohistochemical techniques with antibodies against bromodeoxyuridine (BrdU), PC10 proliferating cell nuclear antigen (PCNA), Factor VIII, and alpha smooth muscle actin (SMA) using standard techniques.

6. **EXPERIMENTAL PROCEDURE**

Ten (10) animals will be included in the study. One (1) animal per treatment

1) bFGF __µg/ml, 10 myocardial injections, each 0.1 cc
2) VEGF __µg/ml, 10 myocardial injections, each 0.1 cc
3) CAM pass BP (Lot #093098), 10 myocardial injections, each 0.1 cc
4) CAM marginal BP (Lot #120498), 10 myocardial injections, each 0.1 cc
5) High MW Povidone, 70%, 10 myocardial injections, each 0.1 cc
6) Low MW Povidone, 70%, 10 myocardial injections, each 0.1 cc
7) High MW Povidone, 50%, 10 myocardial injections, each 0.1 cc
8) Low MW Povidone, 50%, 10 myocardial injections, each 0.1 cc
9) High MW Povidone, 1%, 10 myocardial injections, each 0.1 cc
10) Low MW Povidone, 1%, 10 myocardial injections, each 0.1 cc

Following growth factor or control myocardial injections. the animals will be allowed to recover. Final clinical endpoints assessment will be performed six weeks following growth factor administration.

7   **DURATION OF STUDY**

Four (4) months is the estimated duration of the study including all surgical procedures.

diagnostic tests and histological evaluation.

8. PERFORMANCE EVALUATION

Clinical endpoints include histological analysis to visualize myocardial blood vessel morphology and number.

9. REPORTING

Six (6) months following the start date, Columbia University will provide Sulzer Innotec a final report including all diagnostic and histological data collected during the study.

10. TEST FACILITY

Columbia University
College of Physicians and Surgeons
630 W. 168th St.
New York, NY 10032
USA

11. SPONSOR

Sulzer Biologics, Inc.
Sulzer Carbomedics Inc.
1300B East Anderson Lane
Austin, TX 78752
USA

12. ESTIMATED START DATE

███████████████

13. ESTIMATED COMPLETION DATE

███████████████

PROTOCOL APPROVAL

TEST FACILITY

Columbia University

David Ruby

Study Director

███████
Date

SPONSOR

Sulzer Biologics, Inc.

Program Director

███████
Date

A Growth Factor Mixture That Significantly Enhances Angiogenesis in Vivo

WILFRIED ROETHY, EDUARD FIEHN, KOTARO SUEHIRO, ANGUO GU, GENG HUA YI, JUICHIRO SHIMIZU, JIE WANG, GEPING ZHANG, JOHN RANIERI, RAMA AKELLA, SARAH E. FUNK, E. HELENE SAGE, JAMES BENEDICT, and DANIEL BURKHOFF

Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York (W.R., E.F., K.S., A.G., G.H.Y., J.S., J.W., G.Z., D.B.); Sulzer Inc., Austin, Texas (J.R., R.A., J.B.); and Hope Heart Institute, Seattle, Washington (S.E.F., E.H.S.)

Received May 30, 2001; accepted August 1, 2001   This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Studies of therapeutic angiogenesis have generally focused on single growth factor strategies. However, multiple factors participate in angiogenesis. We evaluated the angiogenic potential of a growth factor mixture (GF_m) derived from bovine bone. The major components of GF_m (SDS-polyacrylamide gel electrophoresis, mass spectrometry, and Western blot) include transforming growth factor-$\beta 1$–3, bone morphogenic protein-2–7, and fibroblast growth factor-1. GF_m was first shown to induce an angiogenic response in chorioallantoic membranes. Next, myocardial ischemia was induced in 21 dogs (ameroid) that were randomized 3 weeks later to received GF_m 1 mg/ml (I), GF_m 10 mg/ml (II), or placebo (P) (with investigators blinded to conditions) injected in and adjacent to ischemic myocardium. Dogs were assessed 6 weeks later using quantitative and semi-quantitative measures. There were GF_m concentration-dependent improvements in distal left anterior descending artery (LAD) opacification by angiography (P: 0.4 ± 0.2, I: 1.1 ± 0.14,

II: 1.6 ± 0.3, angiographic score p = 0.014). Histologically, there was also concentration-dependent vascular growth response of relatively large vessels (P: 0.21 ± 0.15, I: 1.00 ± 0.22, II: 1.71 ± 0.18, vascular growth score p = 0.001). Resting myocardial blood flow (colored microspheres) was not significantly impaired in any group. However, maximum blood flow (adenosine) was reduced in ischemic territories and did not improve in GF_m-treated hearts. GF_m, a multiple growth factor mixture, is a potent angiogenic agent that stimulates large vessel growth. Although blood flow did not improve during maximal vasodilatory stress, large intramyocardial collateral vessels developed and angiographic visualization of the occluded distal LAD improved significantly. The use of multiple growth factors may be an effective strategy for therapeutic angiogenesis provided a more effective delivery strategy is devised that can achieve improved maximum blood flow potential.

Since their discovery over 15 years ago, several angiogenic growth factors have been isolated and purified (Folkman and Klagsbrun, 1987). The concept that such agents could be used therapeutically to induce both small and large vessel growth (angiogenesis and arteriogenesis, respectively) in states of chronic ischemia due to atherosclerosis has driven both experimental and clinical studies. In both ischemic limbs and heart muscle, the majority of studies have focused on the delivery of a single growth factor to stimulate vascular growth. The most extensively studied proteins are members of the VEGF and FGF families (Isner et al., 1996; Mack et al., 1998; Laham et al., 1999; Unger et al., 2000). There is evidence that delivery of these compounds, in the form of protein or gene therapy, can enhance blood flow to ischemic tissue in various experimental models (Unger et al., 1994; Mack et al., 1998). However, for a number of acknowledged reasons, re-

sults obtained in experimental models often do not translate directly into clinical practice.

One unanswered question about therapeutic angiogenesis is whether delivery of a single growth factor to an ischemic organ will be sufficient to induce growth of relatively large conduit vessels (arteriogenesis). Since ischemic syndromes resulting from arteriosclerosis affect the conduit vessels and not the small arterioles or capillaries, the growth of large vessels can be considered requisite for successful therapy. Many factors participate in the process of arteriogenesis (Beck and D'Amore, 1997). Recent studies have identified synergistic effects of angiogenic agents in the induction of vascular growth (Gajdusek et al., 1993; Ramoshebi and Ripamonti, 2000). Moreover, while prior published animal studies using single-factor strategies noted above have shown improvements, none has shown normalization of blood flow to treated ischemic myocardium. Identification of the factors involved, clarification of the role each factor plays,

ABBREVIATIONS: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HPLC, high-pressure liquid chromatography; IBP, inactive bone protein; PAGE, polyacrylamide gel electrophoresis; CAM, chorioallantoic membranes; GF_m, growth factor mixture; Df, fractal dimension; LAD, left anterior descending artery; BrdU, bromodeoxyuridine; CMS, colored microspheres.

and an understanding of the events involved in the process of arteriogenesis currently constitute areas of active investigation (Carmeliet, 2000).

Recently, a naturally occurring growth factor mixture (GF_m) isolated from bovine long bones was shown to enhance bone formation in several models, including a standard rabbit model of lumbar spinal fusion (Boden et al., 1997). Early in the course of investigating the bone formation stimulation properties of GF_m, it became evident that this mixture also promoted vascular growth. This was particularly evident in a rat ectopic bone development assay in which a collagen disk saturated with GF_m was placed subcutaneously over the chest wall. By 3 weeks after implant, the collagen was replaced with a bony ossicle, and an extensive vascular network (including large vessels) was observed on the surface of the ossicles. In the present study, we follow up on these preliminary findings by formally studying the angiogenic properties of GF_m. We present results indicating that indeed this multiple growth factor strategy effectively induces large vessel growth in chronically ischemic myocardium.

Materials and Methods

GF_m. The proteins found in GF_m are purified as one mixture from a noncollagenous protein extract of bovine femurs as detailed previously (Poser and Benedict, 1994). Briefly, mid-diaphyseal segments of bovine femurs are cleaned, pulverized, and demineralized. Proteins of apparent molecular weights between 10,000 and 100,000 are extracted by ultrafiltration, lyophilized, and purified by reverse-phase high-pressure liquid chromatography (HPLC). The protein mixtures containing active ingredients for bone formation used in this study have been shown to elute between 35.0 and 37.1% acetonitrile (v/v) (Poser and Benedict, 1994). Fractions eluting at slightly higher volume percentages have been shown to be inactive for bone formation (inactive bone protein, IBP) and were used in the present study as negative controls.

GF_m Content Analysis. One- and two-dimensional polyacrylamide gel electrophoresis (PAGE), HPLC, mass spectrometry, and Western blot analyses were used to investigate the contents of GF_m. PAGE analysis was conducted according to standard techniques. Tropomyosin (33 kDa, pI 5.2) and lysozyme (14 kDa, pI 10.5–11) were added to the samples as internal markers. Gels were stained with Coomassie Blue, and protein spots were excised from dried gels for mass spectrometry and sequence analysis.

For mass spectrometry, individual fractions from the HPLC column used to isolate the GF_m components from all the bone proteins were separated by SDS-PAGE in the Xcell II minigel system (Novex, San Diego, CA). Bands identified by staining with Coomassie Blue were excised and subjected to trypsin digestion in the gel slice as previously described. Proteolytic fragments were extracted in 50% acetonitrile/0.01% trifluoroacetic acid and were dried. Subsequently, the peptides were dissolved in matrix solution (10 mg/ml 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) containing angiotensin and bovine insulin as internal standards. Samples were spotted onto a sample plate, washed with water to remove buffer salts, and analyzed by a Voyager DE-RP mass spectrometer in the linear mode (Applied Biosystems, Foster City, CA).

For Western analysis, proteins in the bone extract were separated by SDS-PAGE and were electroblotted onto a polyvinylidene difluoride membrane. The blots were probed with either commercially available monoclonal antibodies against various human proteins or with a polyclonal antibody against bovine FGF-1. The bands were visualized with an horseradish peroxidase-conjugated secondary antibody with a chemiluminescent substrate (Pierce Chemical, Rockford, IL) according to standard procedures.

Studies in Chorioallantoic Membranes (CAM). The angiogenic activity of GF_m was compared with that of a range of concentrations of recombinant human bFGF (10 μg/ml) and VEGF (10 μg/ml) or their combination (5 μg/ml each) (proteins obtained from R & D Systems Inc., Minneapolis, MN), as well as their carrier (povidone), in quail CAMs ($n = 6$ per group) (Parsons-Wingerter et al., 2000). Previous studies have shown that vascular growth response in CAMs to either bFGF or VEGF at concentrations of 10 μg/ml are on the plateau of the respective dose-response curves (Parsons-Wingerter et al., 1998, 2000). Fertilized Japanese quail eggs (*Coturnix coturnix japonica*) were opened into Petri dishes on day 3 postfertilization. After 4 days in culture at 37°C (i.e., day 7), the growth factors were solubilized in prewarmed carrier solution and added in a total volume of 0.5 ml to each embryo. The test material was evenly distributed on the surface of the CAM, which was cultured for 24 h at 37°C. Embryos were fixed in 4% paraformaldehyde/2% glutaraldehyde solution in phosphate-buffered saline. The CAMs were dissected from the embryos and were mounted on glass slides. Digital images were acquired at 10× magnification with a computer-supported digital camera attached to a microscope. The fractal dimension (Df) of each image was determined with previously validated software (Parsons-Wingerter et al., 1998). The Df (baseline) for a day 7 CAM is 1.372 (Parsons-Wingerter et al., 1998). The amount of vascular growth in CAMs treated for 24 h was reported as the percentage of change in Df relative to control (povidone-treated) CAMs.

Pilot Study in Canine Myocardium. Six adult mongrel dogs (20–25 kg) were anesthetized with thiopental sodium (15 mg/kg, i.v.) and maintained with 0.5 to 2.0% inhaled isoflurane. Via a left lateral thoracotomy, the proximal left anterior descending artery (LAD) was isolated and an ameroid constrictor was placed to induce ischemia over time. The dogs received intramyocardial growth factor injections of GF_m diluted in povidone (1 mg/ml, $n = 4$) or IBP (1 mg/ml; $n = 2$). All injections were 0.15 ml and were spaced ~1 injection/cm^2. Injections were made in both the anterior (ischemic) and posterior (normal) walls with five to nine injections performed in each region. Animals survived for either 2 or 6 weeks. All animals received postoperative bromodeoxyuridine (BrdU) injections (schedule provided below). Dogs were euthanized with pentobarbital (100 mg/kg), hearts were removed, and transmural tissue blocks were submitted for histologic and immunohistochemical evaluation (Masson's Trichrome stain; BrdU; smooth muscle actin; von Willebrand factor).

Efficacy Study in Chronically Ischemic Canine Myocardium. A randomized, blinded, placebo-controlled study was performed. An ameroid constrictor was placed in 21 adult mongrel dogs (20–30 kg) to create chronic ischemia. To minimize collateral flow in the acute setting, all visible epicardial obtuse marginal or posterior branches seen to connect with the LAD or LAD diagonal vessels were ligated (4–0 polypropylene sutures). One silicon tube (Tygon, Cardiovascular Instrument Corp., Wakefield, MA) was chronically implanted into the left atrium and another into the descending aorta.

Three weeks after ameroid constrictor placement, myocardial blood flow was measured at rest and during adenosine infusion. The chronically implanted aortic line was connected to a transducer (Statham Instruments, Inc., Oxnar, CA) for instantaneous and mean aortic pressure measurement. Colored microspheres (CMS; Dye-Trak, Triton Technology Inc., San Diego, CA) were infused rapidly into the left atrium (2 ml, 6×10^6 spheres) through the left atrium catheter. Withdrawal of a reference blood sample from the aortic line was begun just prior to CMS infusion (7 ml/min for 2 min). Adenosine was then infused at a dose titrated to induce ~20% decrease in mean aortic pressure, followed by infusion (and aortic reference sample withdrawal) of a second set of CMS.

Dogs were then anesthetized as above for a second surgical procedure 3 weeks later. Following anesthesia, a baseline coronary angiogram was performed to confirm ameroid closure and to define the degree of collateral filling of the distal LAD. The ameroid completely occluded flow to the distal LAD in every case. A thoracotomy was then performed and animals were randomized into one of three groups (seven dogs per group): placebo (povidone only), low concentration GF_m (1 mg/ml), or

high concentration GF_m (10 mg/ml). Randomization occurred on the morning of the surgery prior to knowledge of the angiogram and all investigators were blinded to treatment group until the final analyses of all data were complete. Animals received a total of 15 to 20 intramyocardial injections to the LAD area with the test solution (0.15 ml/ injection, one injection/cm^2). Shallow 4–0 Prolene stitches were placed over each injection site to allow their identification at sacrifice. Animals received subcutaneous injections of BrdU starting the day before surgery (25 mg/kg), on the day of surgery, and days 1, 3, 5, 7, 9, 13, and 20 after surgery (15 mg/kg).

Six weeks after treatment, blood flow assessment (CMS) and coronary angiography were repeated. Animals were sacrificed and the heart removed. Three transmural tissue blocks, each containing one or two injection sites, were isolated, cut into epicardial, midwall, and endocardial sections, and evaluated histologically (Masson's Trichrome, BrdU, von Willebrand factor, and smooth muscle actin). The remainder of the heart was divided into 36 transmural blocks; each block was divided further into epicardial and endocardial sections (~1 g each). Blood flow was calculated from the CMS data according to standard techniques (Kowallik et al., 1991).

Statistics. Data were expressed as mean ± S.E.M. Between-group comparisons of ordinal data were performed with a Kruskal-Wallis test followed by repeated Mann-Whitney U tests to determine individual differences. Within-group differences were tested by the Friedman test; if significant, this was followed by repeated Wilcoxon tests. Between-group comparisons of continuous variable were done by one-way analysis of variance followed by Scheffe post hoc test. Within-group comparisons were done by paired samples t test. $p <$ 0.05 was considered statistically significant.

Results

GF_m Composition. PAGE analysis of GF_m included 13 major bands, representing 65% of the protein by weight. These major bands have been identified by mass spectrometry. Two major components are BMP-3 and TGF-β-2. Other identified proteins that are probably not contributors to angiogenic activity include three that are related to histone H1.1 and three that matched with the ribosomal proteins S20, L6, and L32. Also identified were cathepsin L and proteins related to α-2-macroglobulin receptor-associated protein, retinoic acid receptor responder protein 2, secreted phosphoprotein 24, and lysyl oxidase-related protein.

By immunoblotting we have confirmed the presence of BMP-2 through 7, TGF-β-1 through 3, and FGF-1. With the exception of BMP-3 and TGF-β-2, these components are present at less than 1% of the total protein. VEGF and FGF-2 have not been detected.

Quail CAM Assay. Compared with povidone alone, CAMs exposed to GF_m for 24 h showed a greater vascular density and more vessel branchings (Fig. 1, A and B). The rate of vascular growth in CAMs subjected to povidone alone was similar to that of control CAMs (data not shown) and was set at 100% (Fig. 1C). The rate of vascular growth was approximately doubled with bFGF, VEGF, or their combination. GF_m at concentrations of 1 or 10 mg/ml elicited a slightly greater (although not statistically significant) vascular growth relative to that stimulated by bFGF, VEGF, or their combination.

Pilot Study. In GF_m (1.0 mg/ml)-treated animals allowed to survive for 2 weeks, large, BrdU-positive, conduit-sized vessels with diameters up to 300 μm could be detected in areas surrounding the injection sites, both in ischemic and nonischemic areas of the heart (Fig. 2, A–D). Six weeks after treatment with GF_m, numerous well organized large vessels as well as smaller arterioles and capillaries in the surround-
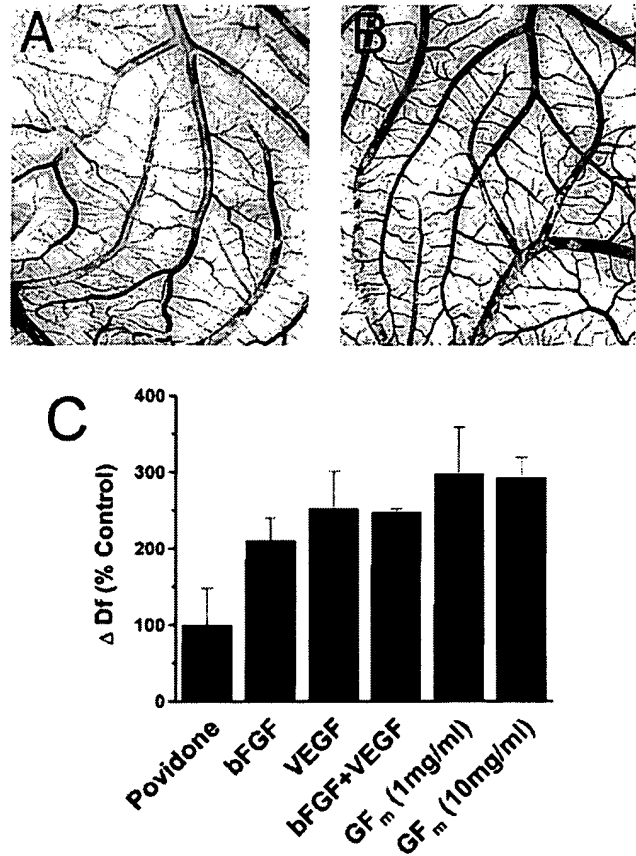

Fig. 1. Effects of growth factors on vascular growth on CAMs of fertilized Japanese quail eggs. Representative digitized pictures of CAMs exposed to either placebo (A) or GF_m 10 mg/ml (B). C, summary of quantitative image analysis of percentage of change in fractal dimension in response to different growth factors (Df, mean ± S.D., n = 6 in each group).

ing myocardium exhibited abundant BrdU incorporation (Fig. 2, E–H). Compared with nontreated ischemic tissue, GF_m-treated ischemic myocardium exhibited a greater than 5-fold increase in BrdU-stained, newly formed arterioles, with diameters ≥50 μm containing ≥2 BrdU-positive cells (6.6 ± 4.0 versus 1.3 ± 1.8 vessels/cm^2, $p <$ 0.05). In contrast, IBP (1.0 mg/ml, an inactive protein fraction derived from bovine bones) failed to induce any significant growth of vessels larger than capillaries either close to the injection sites or in remote areas after 2 weeks (Fig. 3, a and b), although the inflammatory response at the injection site appeared similar with all the factors. These observations suggest that GF_m can induce large vessel growth and the major component is not due to inflammation.

Effects in Chronic Myocardial Ischemia. Histologic samples were examined and graded for vascular growth *outside the scar observed at the injection site* according to a semiquantitative overall vascular growth index (0, no angiogenic response; 1+, mild-to-moderate angiogenic response; 2+, significant angiogenic response). According to this analysis, as summarized in Table 1, GF_m induced a robust concentration-dependent neovascular response in ischemic canine myocardium. The size of the scar was also concentration-dependent.

Baseline and follow-up angiograms were also compared by a three-point semiquantitative scale (0, no change in distal LAD opacification; 1+, mildly improved distal LAD opacifi-
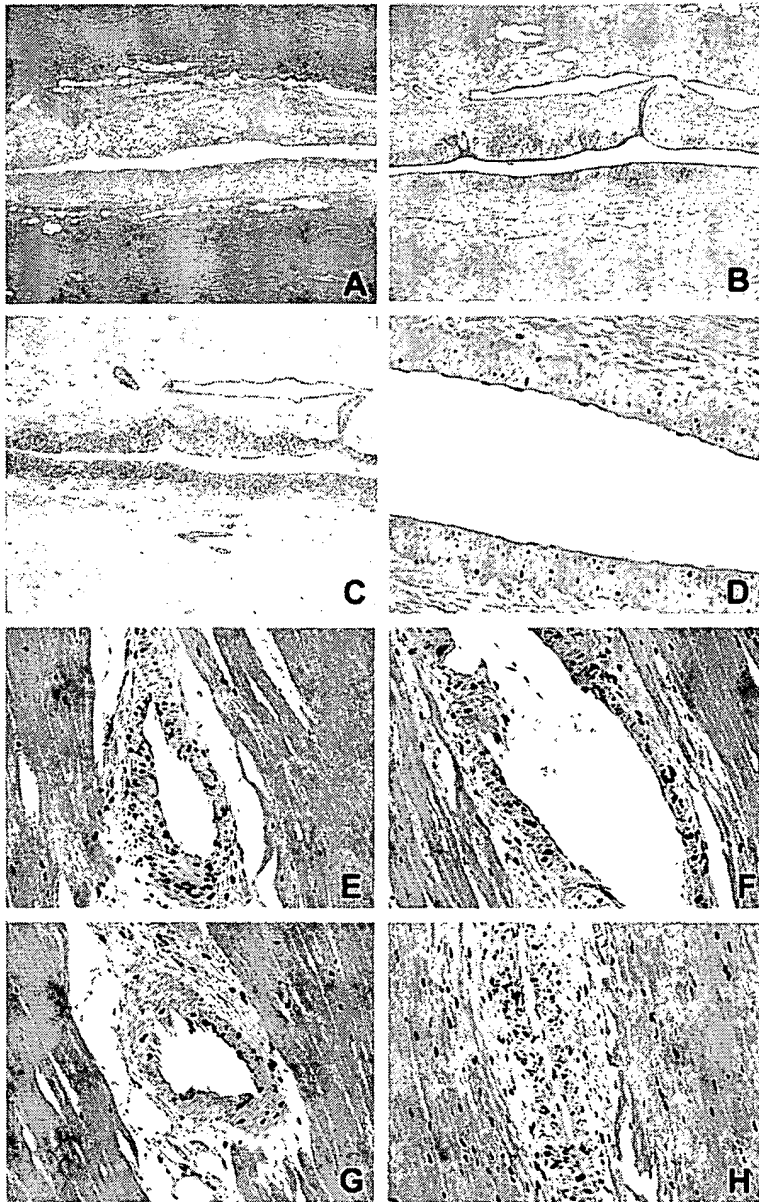
Fig. 2. Masson's Trichrome-stained section (A, 40× original magnification) showing large vessel with several side branches 2 weeks after GF_m injection into ischemic myocardium. As shown in adjacent sections, immunostained with antibodies against von Willebrand factor (B) and smooth muscle actin (C), the vessel is lined by endothelium surrounded by poorly organized layers of smooth muscle cells. That von Willebrand factor staining was not limited to the endothelial layer provides another indication that this vessel was not fully organized. Nuclei staining for proliferating cell nuclear antigen (200×, D) is indicative of neovascularization. Similar vessels were identified in both the ischemic and nonischemic areas of these hearts. Six weeks after ameroid placement and GF_m injection, relatively large but apparently mature vessels were seen (E–H). Evidence of vascular growth is based on the abundant BrdU incorporation seen in the endothelial and smooth muscle cells.

cation; 2+, significantly improved distal LAD opacification). Examples of in vivo angiograms from a high-concentration GF_m animal are shown in Fig. 4. Early during contrast injection at the baseline study (Fig. 4A), the ameroid completely occluded antegrade flow through the LAD; there was only faint LAD opacification late in the injection (Fig. 4B). Six weeks after GF_m treatment in the same animal, a new collateral vessel was seen early during the contrast injection (Fig. 4C, C1) that became more prominent along with more prominent LAD opacification seen later in the injection (Fig. 4D). These findings were not seen in the placebo group. Post-mortem ex vivo angiography on the same heart (Fig. 5, A and B) illustrates the ameroid closure; early during the contrast injection (Fig. 5A), two collateral vessels (C1, C2) were identified as responsible for distal LAD opacification late during the injection (Fig. 5B). Figure 5D shows an ex vivo angiogram from a placebo animal late during a direct

left main injection. Here, "late" means that the picture is obtained with maximum dye injection to ensure visualization of an image obtained with maximal filling of all vessels that are present. Even under these extreme conditions, the LAD is completely missing. Although the image of Fig. 5B (from a treatment animal) is taken later than the image of Fig. 5A from the same animal, it is not as late as in the comparable injection in Fig. 5D as suggested by the relatively little filling of the circumflex vessel. Although this is an "earlier" image than in Fig. 5D, the LAD is readily seen. These findings indicate collateralization to the fully occluded distal LAD is significantly increased in the treatment compared with the placebo animal. The results of the semiquantitative blinded angiographic analysis (Table 1) confirmed a statistically significant improvement in the angiographic score at both high and low concentrations, compared with the placebo group.

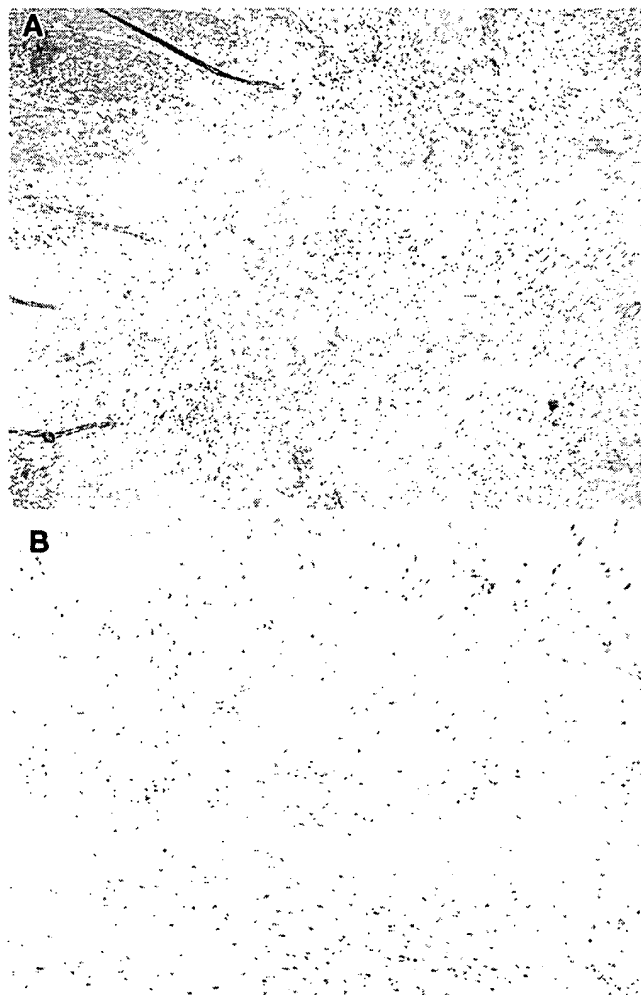Baseline microsphere-derived resting blood flow (3 weeks

Fig. 3. Reactive inflammatory response after treatment with inactive bone protein (IBP, 1 mg/ml) shows scarring with no significant growth of vessels larger than capillaries (a and b, original magnification 40×).

after ameroid placement) was relatively normal in all myocardial segments, with only mild decreases in the anterior wall (Fig. 6; since results from endocardial and epicardial samples were not significantly different, they were pooled for this analysis). During adenosine vasodilation, however, blood flow increased ~4 fold in most segments, but not in those in the anterior region supplied by the LAD distal to the ameroid

constrictor, which increased by less than a factor of 2. These features were similar in all treatment groups, as indicated in Table 1. Thus, despite the histologic and angiographic evidence of large vessel growth, maximal vasodilatory blood flow did not improve following GF_m treatment.

Discussion

GF_m, a protein mixture derived from bovine long bones, is an effective angiogenic agent in quail CAMs and canine myocardium. Several of the identified ingredients of GF_m are among the list of known angiogenic factors, and synergism between some GF_m components (e.g., BMP-7, bFGF, and TGF-β-1) has already been demonstrated in the CAM assay (Ramoshebi and Ripamonti, 2000). In ischemic myocardium, GF_m treatment was associated with growth of vessels with diameters as large as 300 μm with abundant BrdU incorporation. Analysis of the composition of GF_m confirmed the presence of several known angiogenic growth factors, such as bone morphogenic proteins BMP-2 through 7, TGF-β-1 through 3, as well as FGF-1. However, approximately 30% of the protein mass is unidentified. Thus, the observed effects could be related to any one or combination of the known growth factors, or they could reflect activity of as yet unidentified and possibly unknown factors. However, an inflammatory response is not the predominant mechanism underlying the effects of GF_m since similar vascular growth responses were not observed with IBP, which did incite an inflammatory response similar (histologically) to GF_m. However, although not sufficient, this does not exclude the possibility that an inflammatory response is necessary for the angiogenic response of GF_m to occur.

Angiographic findings in the chronically ischemic dog hearts provided additional evidence of large vessel growth. All ameroid constrictors were completely occluded so that at the baseline ischemic evaluation, there was only faint visualization of the distal LAD during contrast agent injections. A GF_m concentration-dependent increase in distal LAD opacification (blinded analysis) was observed, suggesting that collateralization to the distal LAD was improved. This was associated with the appearance of new collateral vessels either bridging around the ameroid constrictor or connecting marginal branches of the circumflex artery to diagonal vessels of the LAD. These vessels, which were particularly evident on ex vivo angiograms, took circuitous courses that are typical of new vessels.

As identified in prior studies of dogs (Unger et al., 1993)

TABLE 1

Summary of quantitative and semiquantitative parameters of vascular growth from histology, angiography, and microsphere analysis of blood flow (mean ± S.E.M., n = 7 for each group for each measurement)

	Placebo	GF_m (1 mg/ml)	GF_m (10 mg/ml)	p
Overall vascular growth index	0.21 ± 0.15	1.00 ± 0.22*	1.71 ± 0.18*, **	0.001
Scar (mm²)	6.5 ± 1.9***	8.7 ± 2.2	15.9 ± 3.4	0.047
Angiographic score	0.4 ± 0.2	1.1 ± 0.14*	1.6 ± 0.3*	0.014
Baseline resting blood flow (ml/min/g)[a]	0.76 ± 0.06****	0.91 ± 0.17	0.90 ± 0.10	
Baseline maximal blood flow (ml/min/g)[a,b]	1.66 ± 0.18****	1.83 ± 0.19****	1.79 ± 0.15****	
Resting blood flow 6 wk after treatment (ml/min/g)[a]	0.70 ± 0.05****	1.07 ± 0.25****	0.90 ± 0.12****	
Maximal blood flow 6 wk after treatment (ml/min/g)[a,b]	1.90 ± 0.18****	2.21 ± 0.12****	1.93 ± 0.19****	

* $p < 0.05$ vs. placebo; ** $p = 0.051$ vs. GF_m 1 mg/ml; *** $p < 0.05$ vs. GF_m 1 mg/ml; **** $p < 0.05$ vs. control area.
[a] Blood flow measurements are from the anterior ischemic region.
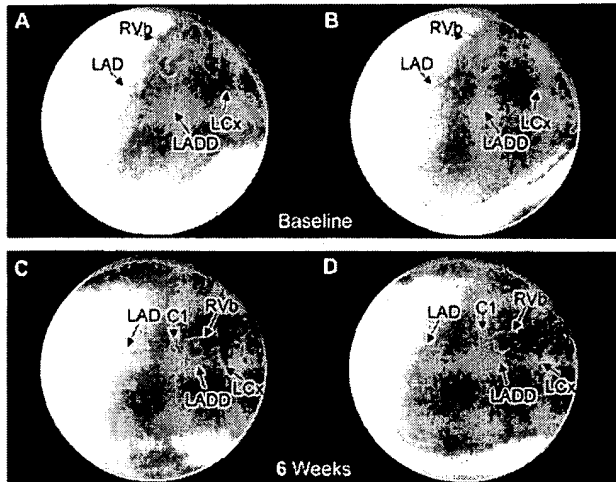[b] Maximal blood flow achieved by intravenous adenosine.

Fig. 4. Sequence of in vivo angiograms from a dog's heart at baseline (3 weeks after ameroid constrictor placement; A and B) and 6 weeks after treatment (C and D) with GF$_m$ (10 mg/ml). At baseline, the ameroid was completely occluded and the LAD demonstrates minimal filling, both early (A) and late (B) during the contrast injection. Six weeks after treatment, the ameroid was again seen to completely occlude the LAD (C), but the LAD showed complete reconstitution late in the injection (D). A newly formed collateral (C1) was detected surrounding the experimentally occluded segment. LADD, diagonal branch; LCx, circumflex artery; RVb, right ventricular branch.
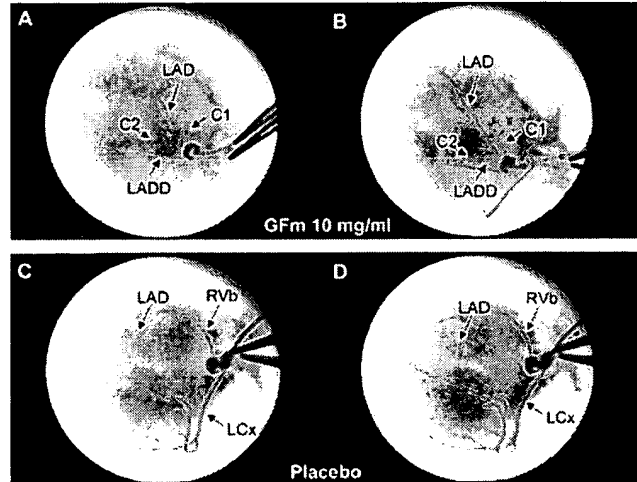
Fig. 5. Sequence of representative ex vivo angiograms taken at the terminal experiment 6 weeks after treatment with GF$_m$ (10 mg/ml; A and B) or povidone (placebo; C and D). The contrast agent was injected via a catheter engaged in the left main coronary artery, revealing the left circumflex coronary artery (LCx) and a right ventricular branch (RVb). Ameroid constrictors completely occluded the LAD in both cases. After GF$_m$ treatment, the LAD opacified early during the injection (A) via two collateral vessels (C1 and C2). Later during the injection (B), the entire LAD was seen. With placebo, the distal LAD is not seen early (C) and is barely evident late during the injection (D).

and pigs (Giordano et al., 1996), resting blood flow 3 weeks after ameroid constrictor placement was not significantly decreased compared with the normally perfused region, the result of natural collateralization. Since distal LAD opacification was poor at this time point, it is evident that normalization of resting flow is due to natural angiogenesis that occurs in the setting of chronic ischemia (Ware and Simons, 1997). However, despite the histologic and angiographic findings indicating the presence of large vessel growth, blood flow

to the anterior wall was not improved during vasodilatory stress induced by adenosine. Distal LAD opacification on resting angiography does not in any way indicate the maximal blood flow capacity into the vascular bed; it merely indicates that new anatomic connections exist. This is completely analogous to the common clinical experience where, although a totally occluded epicardial vessel can frequently be visualized angiographically by flow of contrast agent from
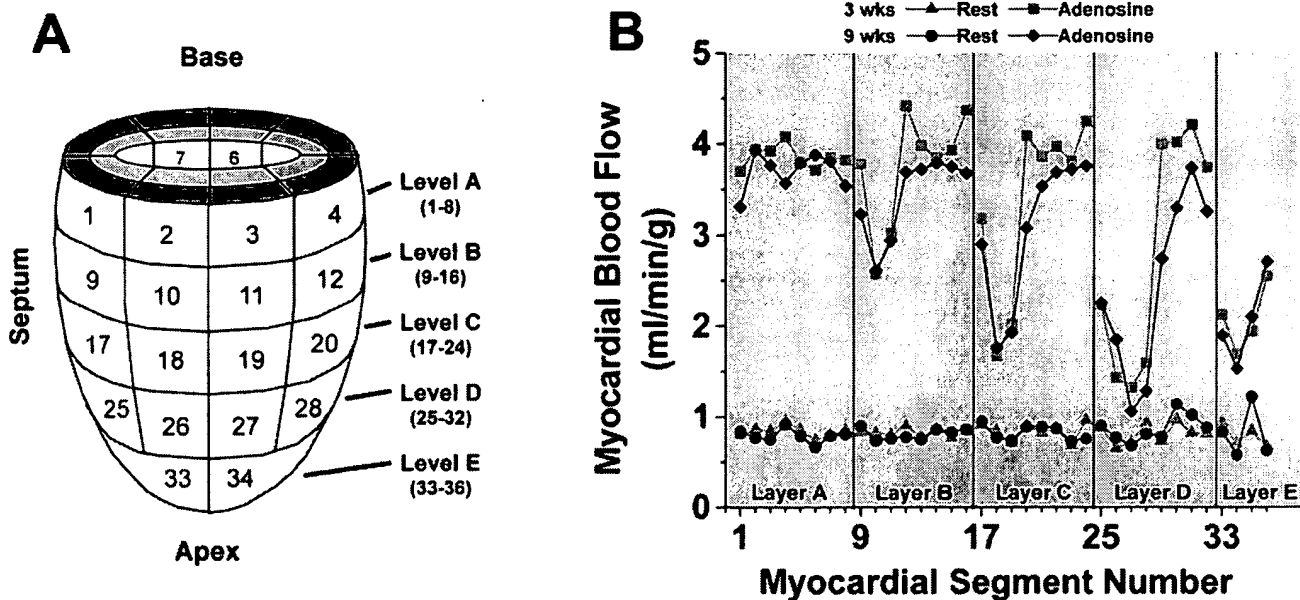

Fig. 6. Hearts were cut into 5 layers (A-E, base to apex) to produce 32 transmural blocks (A). Blood flow at each time point at rest and during vasodilation (adenosine) of each segment was plotted as a function of segment number to reveal ischemic and normally perfused segments (B). This result, obtained from a dog treated with GF$_m$ 10 mg/ml, shows no significant difference between baseline and 6 weeks after treatment. Results from endo- and epicardial layers (analyzed separately) were subsequently pooled because they were highly similar.

collateral vessels, a myocardial perfusion defect is typically observed during stress on a nuclear perfusion imaging study.

Thus, while GF_m effectively induces large vessel growth, it could be that the delivery strategy (direct mid-myocardial injections at ~1-cm interinjection spacing) may not be optimal for improving maximal blood flow to ischemic myocardium. Use of a delivery method to ensure that new vessels grow from well perfused normal epicardial vessels neighboring the ischemic territory [e.g., an intra-arterial injection strategy (Giordano et al., 1996) or preferential subepicardial delivery] might be more effective.

The application of single exogenous growth factors either as protein or gene therapy has thus far been the standard in clinical trials of therapeutic angiogenesis. However, angiogenesis is not a process induced, sustained, or completed by a single molecule (Folkman and Klagsbrun, 1987; Beck and D'Amore, 1997; Coussens et al., 1999; Carmeliet, 2000; Carmeliet and Jain, 2000). Published preclinical studies of single growth factor strategies, whether with proteins or gene therapies, whether with FGF or VEGF, whether injected into the muscle or into an artery, have never demonstrated complete normalization of blood flow to the ischemic territory; such results are summarized in recent reviews (Ware and Simons, 1997; Simons et al., 2000). Clinically, although results of a few small (some unblinded) studies of single growth factor strategies have provided encouraging results (Schumacher et al., 1998; Vale et al., 2001; Laham et al., 1999), results from two relatively large-scale multicenter, double-blind studies now available have been negative; specifically, the VIVA trial of intracoronary and intravenous VEGF (Henry et al., 1999) and the FIRST trial of intracoronary bFGF (M. Simons, unpublished observations). Even in the unblinded clinical study, the extent of revascularization was incomplete, as has been observed in the animal studies. There are multiple possible explanations for variable results between animal studies and for the negative results reported in the earlier noted clinical trials. The fact that single growth factor strategies were used is among the possibilities (mode of delivery being another important factor), but it would be premature to conclude at this time that this is the decisive factor.

Conclusions. The choice of growth factor(s) and delivery strategies for therapeutic angiogenesis are currently the topic of intensive research. Some clinical trials based upon positive preclinical studies have provided negative results (see for example, Henry et al., 1999; Simons et al., 2000) warning about the potentially limited power of preclinical studies to predict clinical utility. Nevertheless, the present results indicate that GF_m, a mixture of growth factors, induces the formation of relatively large vessel. Lack of improved blood flow in treated animals does not diminish the importance of the histologic images of large new vessels seen in response to GF_m treatment. This does highlight the fact, however, that there are limitations to the overall strategy used in the present study. This could relate to the use of an intramyocardial route of administration, the density of injections, the dose of GF_m, or other unidentified factors that may also have impacted the results. As such, the present results serve to emphasize the point that, although necessary, it is not sufficient for an angiogenic therapy to induce vascular growth. The overall strategy, which includes the substance and its mode of delivery, must ensure that the new vessels become part of an effective vascular bed.

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